INTERACTION OF HYDROGELS WITH BIOLOGICALLY RELEVANT SPECIES IN AQUEOUS PHASE

Final report
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Poly(N-isopropylacrylamide) (PNIPA) hydrogels are widely known for their reversible temperature-induced discontinuous volume phase transition (VPT) at the lower critical solution temperature (LCST) around 34 °C. The majority of thermosensitive gels used in biotechnological applications are either homopolymers or copolymers of *N*-isopropylacrylamide (NIPA). Fields of application of these hydrogels include drug delivery systems, actuators, chemo-mechanical devices, chemical switching, temperature-modulated extraction, affinity precipitation, bioseparation, construction of sensors, enzyme immobilization, immunodiagnostics, gene therapy, thermoresponsive self-assembling micelles, photo switching. These applications are based on the ability of PNIPA hydrogels to adsorb, retain and separate different target molecules.

The nature of the interactions between small foreign molecules and larger structures, e.g., biological membranes, proteins or swollen gels, is a vital indicator in understanding a wide variety of systems of biomedical interest.

1. Studies on PNIPA hydrogels

1.1. The synthesis process

PNIPA hydrogels were prepared in water using a redox initiator. The copolymer composition at high conversion (> 95%) was determined indirectly by high performance liquid chromatography (HPLC) analysis of the leaching water and directly by solid state ^{13}C CP MAS NMR (cross polarization magic angle spinning nuclear magnetic resonance) spectroscopy of the dried gels, and was found to be close to that of the feed. The effect of cross-linker *N*,*N*"-methylene-bisacrylamide (MBA) content in the copolymer was investigated in the concentration range of 1.1–9.1 mol% on the rheological behaviour and mechanical strength of the hydrogels. Gels with very low cross-linker content (1.2–1.5 mol% MBA) have a very loose network structure, which is significantly different from those with higher cross-linker content manifesting in higher difference in storage modulus. The gel with the highest cross-linker content (9.1 mol% MBA) behaves anomalously due to heterogeneity and the hindered conformation of the side chains of PNIPA [1].

1.2. Microphase structure of poly(N-isopropyl acrylamide) hydrogels

Numerous applications of PNIPA rely on the stimuli response of PNIPA. Its rate of deswelling, however, is extraordinarily slow. In contact with excess solvent, the volume of the unstable intermediate structure can be conserved for many days, even for samples as small as one millimetre in size. Measurements using small and wide angle X-ray scattering (SAXS/WAXS) and pulsed field gradient NMR revealed that in the microphase separated state in PNIPA hydrogels the cavities occupied by the water phase form a connected network of channels of submicron cross-section. The connectivity of the cavities implies that the overall gel structure is an irregular sponge phase. The absence of compartmentalisation of the water phase implies that the slow deswelling rate of the gel is not due to trapping of the water. Within the polymer-rich phase of the gel, water occupies about two thirds of the volume. It possesses two main components, a majority group moving in narrow channels, and a weakly mobile minority group associated with the water of hydration. The diffusion coefficient of the latter is about two orders of magnitude smaller than that in the water phase. Neither group is localized [2, 3].

1.3. Deswelling kinetics of PNIPA hydrogels

The importance of PNIPA as responsive materials has inspired numerous investigations into their swelling/deswelling kinetics. At the molecular level, however, the kinetics of this system remains

incompletely understood. Our results on the rate of deswelling of PNIPA gels using X-ray photon correlation spectroscopy (XPCS) and observations of the time dependence of the macroscopic deswelling (***Figure 1***) support the notion that the ultraslow deswelling rate observed at the volume phase transition in PNIPA hydrogels is the result of jamming. This phenomenon is intrinsic to the polymer structure and is not attributable to its hydrophobic character in the high temperature regime. It appears that structural inhomogeneities in the gel, such as those associated with cross-links, release the constraints of jamming and facilitate rapid relaxation [4, 5].

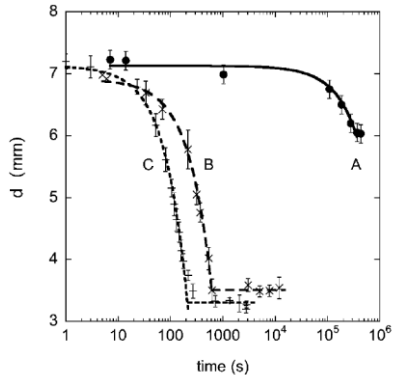

Figure 1. *Time dependence of the diameter d of PNIPA hydrogel disks during macroscopic deswelling at 50°C for samples of increasing cross-link density A, B and C. Lines are fits of the data to* $d=d_0 - vt$*, where v is the velocity of the disk at its outer diameter*

1.4. Influence of salts and buffers (pH) on the phase transition properties of PNIPA hydrogels

The phase transition temperature of PNIPA hydrogel reduces in the presence of salts. The effect is proportional to the concentration and influenced by the chemical nature of the ions, following the Hofmeister series in both the cationic and anionic range. Also, according to Hofmeister's conclusions, anions results in a higher shift than the cations. Although PNIPA is considered as a neutral gel which - in its native form exhibits only a negligible pH sensitivity -, its phase transition temperature T_{VPT} and surface charge was found to be influenced by the pH of the swelling medium (***Figure 2***). Our comparative studies in phosphate buffers – almost exclusively used when biomedical systems are studied– and aqueous salt solutions of the same anion show, that i) the heat of deswelling (from micro-DSC measurements) is not influenced; ii) the degree of swelling linearly increases with pI (I is the ionic strength) and it depends on the cation; iii) ΔT_{VPT} can be enhanced by i) reducing pI or increasing pH [6, 7, 8].

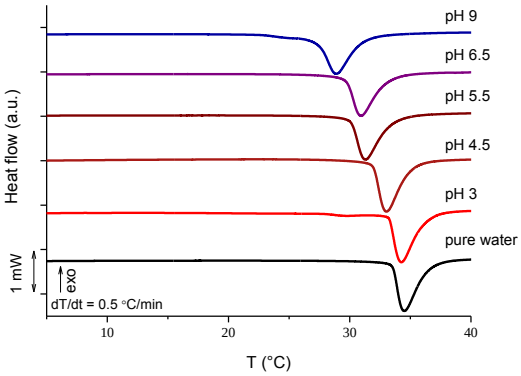

Figure 2*. Micro-DSC response of PNIPA hydrogels in buffer solutions. Heating rate: 0.02 °C/min. Successive curves are shifted vertically for clarity. Interval between graduations on ordinate axis:0.2mW.*

1.5. Influence of small aromatic molecules on the phase transition properties of PNIPA hydrogels

It has been observed that addition of small guest molecules, even at low concentration, influences appreciably the transition temperature of these hydrogels. The mechanism of this temperature shift is unknown. Several aromatic drug models, such as salicylaldehyde, 3,4-dimethoxybenzaldehyde, m- and p-hydroxy-benzaldehyde, ethylvanillin, benzoic acid and methyl-p-hydroxybenzoate decrease the LCST of PNIPA. The shift depends on the chemical structure and the concentration of the additive, but there is no correlation with the hydrophobicity or the solubility of the additive. This suggests that the LCST may be controlled by specific additive - polymer interactions. When a small organic molecule replaces a hydration

water molecule on a given polymer chain, the chain may become more hydrophobic, shifting the LCST toward lower temperatures. The LCST decreases with increasing concentration of the guest molecules. Although PNIPA gels have been extensively investigated in the past decades, their interaction with small molecules in the transition is not yet fully understood. In drug delivery systems, however, it is crucial that release not be inhibited by specific interactions with the polymer network. For this reason it is important to investigate the nature of the interactions between certain guest molecules and PNIPA hydrogels. In this project we studied the interaction between PNIPA and several small aromatic drugs, e.g., phenols, coffein and its analogues, proteins, etc. [6, 9, 10]. Here the interactions of phenols and dopamine (3,4-methylenedioxymethamphetamine) are highlighted. A notable feature of the interaction is that T_{VPT} decreases linearly with the aromatic concentration. ***Figure 3*** shows this behaviour for the same PNIPA gels in solutions of phenol, pyrocatechol, phloroglucinol, resorcinol, and dopamine. The effect of dopamine, which is contrary to that of phenol and pyrocatechol, indicates that dopamine improves the solvent quality.

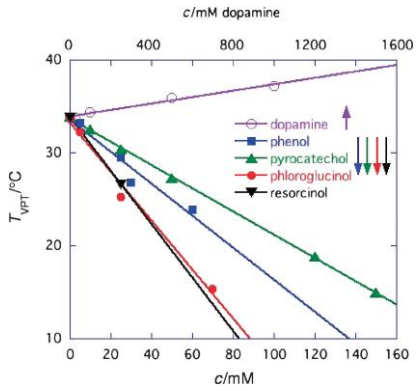

Figure 3. *Dependence of T_{VPT} on guest molecule concentration in pNIPA hydrogels. Note that for dopamine the upper concentration scale applies.*

Earlier we found evidence of the associative behaviour between phenol and PNIPA from small-angle neutron scattering (SANS) measurements [11]. Significantly more direct information on this molecular association is found from two-dimensional proton NMR CRAMPS (combined rotation and multiple-pulse spectroscopy) measurements. Intermolecular cross peaks may appear when the proton-proton distance is less than 1 nm. The appearance of intermolecular cross peaks demonstrates the existence of host-guest interactions (***Figure 4***). Varying the time during which polarization transfer can occur modifies the intensity of the cross peaks. The intermolecular distances can be estimated by rate matrix analysis. Such observations on phenol in PNIPA allowed the proximity distances of methyl-phenol and methyne-phenol to be determined as 5 and 5.5 Å, respectively. These distances are shorter by 0.2-0.3 Å in pyrocatechol. In dopamine, however, no intermolecular cross peaks were observed. This technique is currently being extended to PNIPA hydrogels with other aromatic compounds [12].

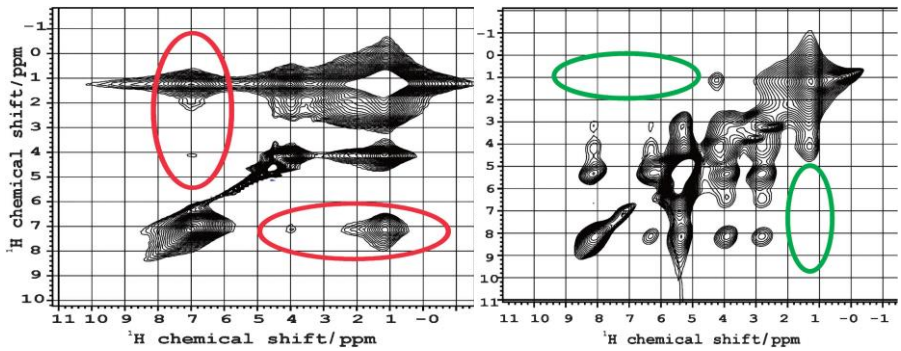

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Figure 4. *^{1}H-^{1}H correlation CRAMPS spectra at 10 kHz spinning rate with 1000 μs of mixing time above VPT. Appearance of intermolecular cross-peaks demonstrates the close proximity in swollen PNIPA gel containing phenol above the VPT (a). The corresponding cross peaks are absent with dopamine (area inside green ellipses) (b).*

Variation of the mixing time - the time during which polarization transfer can occur- modifies the intensities of the cross peaks. A series of correlation spectra was recorded with different mixing times to determine intramolecular distances by rate matrix analysis in the case of phenol and pyrocatechol.

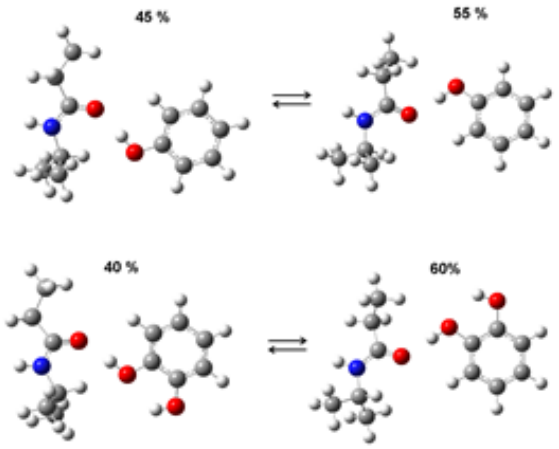

Figure 5*. Optimized geometries of NIPA-phenol and NIPA-pyrocatechol systems with b3lyp311g++(d,p) base. The H-bond is stronger with pyrocatechol, as indicated by the shorter (~0.05 Å) distance Color code: grey – carbon, white – hydrogen, red – oxygen, and blue – nitrogen atoms.*

Although the PNIPA/phenol and PNIPA/pyrocatechol systems are similar, their build-up curves were different. Exact analysis of the experimentally recorded build-up curves is not feasible, but on introducing simplifying assumptions an acceptable solution was found [13]. Density functional theory (DFT) calculations were carried out to investigate possible conformations of these two systems. Comparison of the total energy of their different conformations shows that in the most favorable structures the phenolic compound is attached to the carbonyl group by a H-bond. As the free energy of the phenol and pyrocatechol - amine group conformations is higher by 8-10 kcal/mol, their population is negligible (less than 1%). Although the second-order bond formed is strong, the system has great freedom to rotate around the carbonyl C=O axis. This freedom strongly influences the distance between the side-chain and the phenolic compound. To find the preferred conformations a scan was carried out around the C=O axis using the Gaussian 03 software package. The results showed that the rotation around the axis is free, with two favorable conformations with both aromatics (***Figure 5***). The average side chain - phenol distance is 0.2 Å longer than the side chain - pyrocatechol distance. Although the interaction between the small aromatic molecules and the PNIPA has been observed by several techniques, this is, to our knowledge, the first time that the interacting atoms and the most probable conformations have been specified. Experimental observations based on high-resolution DSC, SANS and high-resolution solid-state NMR converge to show that small aromatic molecules interact with PNIPA hydrogels, not only by altering the average solvent quality of the diluent, but also by specific association with the side-chain groups in the NIPA monomer subunits.

2. Studies on PNIPA co-polymers

2.1. Co-polymers with a crown ether (CE)

An important consideration in many applications is that copolymerization with hydrophilic or hydrophobic monomers is often accompanied by a shift of LCST to higher or lower values. Co-polymerization of PNIPA based thermosensitive hydrogels is also a possible route to enhance the storage capacity of guest molecules. The influence of several co-polymers of PNIPA were synthesized and studied in order to compare their potential in various applications. The hydrophilic - hydrophobic balance of PNIPA can be preserved by a suitable choice of amphiphilic co-monomer. Crown ethers (CE) decorated with aromatic groups are good candidates, since they contain several ethoxy units, the polar nature of which compensates for the hydrophobicity of the aromatic groups. It was found earlier, that the swelling ratio in water decreases with respect to the unmodified gel. The invariance of the elastic modulus implied that the CE was incorporated into the network chains of the gel. The temperature of the VPT was lowered and the transition broadened by the CE [6].

Within the frame of this project PNIPA co-polymers with tuned N^9-propenoil-3,6,12,15-tetraoxa-9,21-diaza-bicyclo [15.3.1] heneicosa-1(21),17,19-triene CE-content were synthesized. T_{VPT} as well as the heat of deswelling was reduced, while the phenol sensitivity was increased by CE (***Figure 6a***). The co-

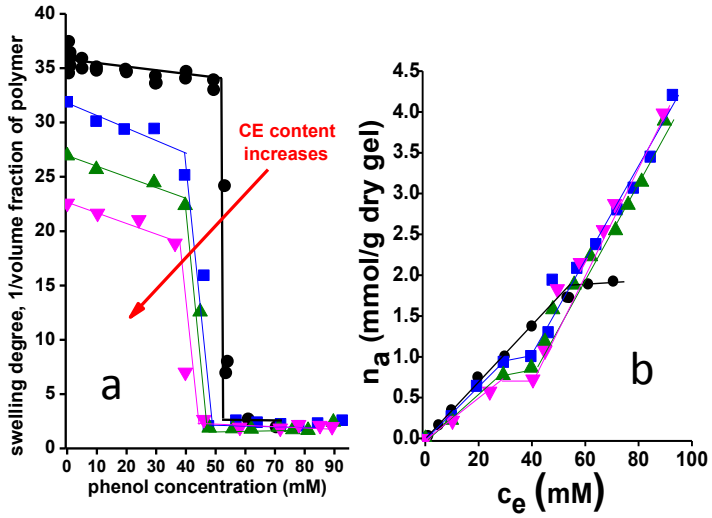

Figure 6*. Influence of the CE content on the swelling in aqueous phenol solutions (a) and on the phenoluptake capacity of the PNIPA/CE co-polymers (b),* 20 °C.
Colour code: NIPA/CE20 (▼), NIPA/CE40 (▲), NIPA/CE60 (■)

polymer not only enhances the phenol uptake but also modifies the mechanism of the sorption as it can be deduced from the shape of the isotherms in ***Figure 6b*** [6].

2.2. Co-networks with polyisobutylene (PIB)

A substantial disadvantage of pure PNIPA gels from practical point of view is its mechanical vulnerability. Amphiphilic co-networks however are mechanically more stable than their pure homopolymeric analogues. These novel, rapidly emerging materials also consist of hydrophilic and hydrophobic polymer components, which are covalently bonded together in a cross-linked macromolecular assembly. Such materials have extraordinary properties, such as swelling with no dependence on solvent polarity, nanophase separated structure, biocompatibility and extreme mechanical strength, which make them applicable in many fields of life and material sciences. PNIPA-*l*-polyisobutylene (PIB) amphiphilic conetwork was synthesized in the Research Centre for Natural Sciences, MTA. DSC investigations performed in our laboratory showed that similarly to CE, the presence of the hydrophobic comonomer reduces T_{VPT} proportionally to its PIB content: the hydrophobic interactions become more favorable in water even at lower temperatures than H-bond formation. In the conetworks the reduction of T_{VPT} is accompanied with a much more gradual progress of the endothermic peak indicating that the phase transition slows down, compared to the homopolymer gel, because the reorganization of the polymer chains/segments is hindered in the conetworks. A reduction in the thermal conductivity of the more hydrophobic system may also contribute to this effect [14].

3. Redox- and pH-responsive cysteamine-modified poly(aspartic acid)

Recently particular attention has been paid to multiresponsive polymers and hydrogels in order to achieve more specific answers to environmental triggers and to approach the complex behaviour of living systems. We focused on the exploitation of the changes in pH and redox potentials. The limited pH-sensitivity of PNIPA was easily exceeded by polyionic polymers. Redox-sensitivity was designed by mimicking the redox processes in living cells, through the thiol-disulfide interconversion within the polymer network. In view of the importance of thiol-disulphide exchange reactions in biological processes, this feature is of direct biological relevance. The synthesis and characterization of pH- and redox-sensitive hydrogels, namely cysteamine-modified polysuccinimide and poly(aspartic acid) were studied. Reversible gelation and dissolution were achieved both in dimethylformamide (DMF) and in aqueous medium via a thiol-disulfide interconversion in the side chain of the polymers. Structural changes were confirmed by Raman microscopy and rheological measurements. The stimuli sensitive properties along with the high water content and good mechanical stability make disulfide cross-linked PASP hydrogels good candidates for human biological applications such as drug delivery systems and implants, as well as for further applications in which in-situ gelation is beneficial. However, their drug release characteristics must be studied in more detail for future exploitation. Beside the proposed polymers, the synthetic method can be applied to various redox-sensitive gels for use as redox actuators in general applications [15].

4. Studies on cryopolymers

A new group of soft matters that attracts interest in biomedical applications is the family of supermacroporous hydrogels. The novelty of these gels is their network architecture, which contains large voids generated by the cryogel preparation technique (***Figure 7***) and which provide for prompt delivery of a specific fraction of the guest molecules. The resulting wide pores of these cryogels can accommodate large molecules and simultaneously provide low flow resistance.

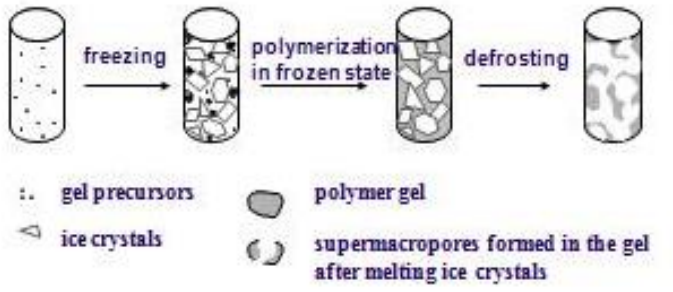

Figure 7. *Cryogel production*

PNIPA/CE and poly(aspartic acid) cryopolymers of various geometry were synthesized in aqueous and organic medium (***Figure 7***). It was found that beside the parameters affecting the 'traditional' gel synthesis, the temperature, cooling rate or the shape of the casting vessel also significantly influence the porosity of the cryogels. The incorporation of the CE at low temperature (-20 °C) was very limited, however, it had a significant influence on the porosity of the walls separating the supermacroporous regions, because its solubility is extremely low in the reaction conditions.

Neither the preparation method nor the limited CE content influenced the T_{VPT} and its enthalpy [16]. The kinetics of the deswelling was the same as with the traditional gel, but reswelling was found to be much faster (***Figure 8***). These trends were very similar in case of PNIPA, PNIPA/CE and the poly(aspartic acid) cryogels. Drug uptake and release tests confirmed that these cryogels have a high potential in this application [17].

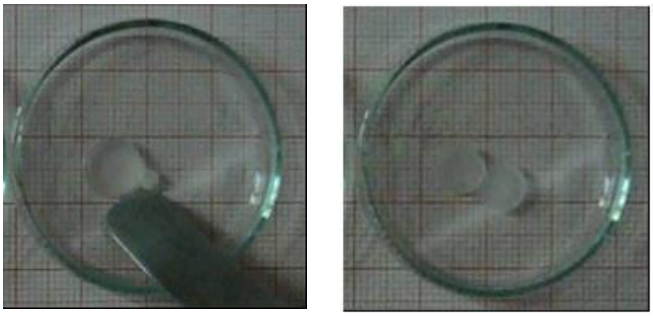

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Figure 8. *A dry(diameter d 3 mm) and a fully swollen cryo-poly(aspartic acid) (d=9 mm) contacting each other in water 14 s (a) and 120 s(b) after contacting them. The swelling/deswelling is significantly faster than int he 'traditional' hydrogels.*

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